Introduction

The protein product of the VHL gene, pVHL, serves many functions, the most well-characterized of which relate to its role in the E3 ligase complex that polyubiquitinates the transcription factor, hypoxia-inducible factor alpha (HIF-α), thereby marking it for proteasome-mediated degradation (1). In this capacity, pVHL plays a central role in mammalian cellular responses to ambient oxygen tension. Biallelic inactivation of the VHL gene characterizes both hereditary and sporadic forms of clear cell renal cell carcinoma (RCC). Approximately 90% of clear cell RCCs manifest biallelic VHL inactivation through genetic and epigenetic mechanisms, whereas nonclear cell RCC variants such as papillary and chromophobe RCCs are devoid of VHL gene alterations and express wild-type pVHL (2).

Reexpression of pVHL suppresses tumor formation in pVHL-deficient murine models (3), and HIF-α expression is required for tumorigenesis in the context of pVHL deficiency (4, 5). Thus, there is a preclinical rationale for drug development aimed at inhibition of the pVHL–HIFα interaction. Unfortunately, efficient and selective restoration of the VHL gene in tumor cells of actual patients is not achievable with current gene therapy technologies, and transcription factors such as HIF-α are not readily amenable to drug development. Therefore, despite the etiologic role of the pVHL–HIFα relationship in renal carcinogenesis, alternative strategies aimed at targeting more traditional “druggable” targets are required.

In the case of clear cell RCC, proto-oncoproteins that are negatively regulated by pVHL may recapitulate the state of nononcogene addiction under the conditions of biallelic VHL inactivation. That is, the identification of biochemical signals, especially “druggable” kinases that are activated in response to VHL loss in a HIF-α-independent fashion could provide alternative opportunities for therapeutic development. Indeed, there is evidence for the existence of HIF-α-independent effects of pVHL. For example, pVHL regulates an inhibitory phosphorylation of caspase recruitment domain 9 protein (CARD9) by CK2 and downregulates activated PKC activity (6, 7). Important genotype–phenotype correlations in the subtypes of von Hippel–Lindau syndrome provide further evidence for HIF-α-independent effects of pVHL. In type 2C von Hippel–Lindau syndrome, the associated VHL mutations...
do not lead to HIF-\(\alpha\) dysregulation, yet patients develop pheochromocytomas (8). Thus, biochemical and clinical correlative evidence points to the existence of HIF-\(\alpha\)-independent effects of pVHL that are germane to oncogenesis. The c-jun-NH\(_2\)-kinase (JNK) is a mitogen-activated protein kinase (MAPK) required for Ras-induced transformation (9). It is protumorigenic in many tumor model systems. Its effects are principally mediated through phosphorylation of members of the AP1 family of transcription factors, such as c-Jun (10). JNK principally mediated through phosphorylation of members of the classical NF-kB pathway, which is constitutively activated in VHL-inactivated clear cell RCC in an HIF-\(\alpha\)-independent manner. Accordingly, we investigated the potential for JNK to function in a nononcogene addiction fashion in the context of pVHL deficiency.

Materials and Methods

Reagents

The isogenic pairs of ACHN, SN12C, 786-0, UOK121, and UMRC6 cell lines have been described (11, 12). The ACHN and SN12C isogenic pairs were obtained from George Thomas (Oregon Health Sciences University, Portland, OR) and were tested for pVHL and HIF-\(\alpha\) expression by Western blotting (Supplementary Fig. S1). The 786-0 cell lines were obtained from W. Kaelin (National Cancer Institute, Bethesda, MD). The 786-0, UOK121, and UMRC6 isogenic pairs of cell lines were originally tested for pVHL and HIF-\(\alpha\) expression by Western blotting as described earlier and are retested quarterly (12). The TRAF6-DN was used as described (13). Lentiviral plasmids designed to express target-specific or non-silencing (NS) short hairpin RNA (shRNA) were obtained from Open Biosystems, as was the pBabe retrovirus was used to express HIF-\(\alpha\) in VHL\(^{-}\) cells (14, 15). The pRL-SV40 Renilla luciferase reporter for normal- and SN12C), the parental cell line expresses endogenous wild-type pVHL and HIF-\(\alpha\) expression. We employed 2 groups of isogenic pairs of RCC cell lines. In one group of RCC cell lines (786-0, UOK121, and UMRC6), the parental cell line manifests biallelic VHL gene inactivation (VHL\(^{-}\)), and pVHL expression is restored in the isogenic counterparts (VHL\(^{+}\)) by stable transduction of a retrovirus expressing the wild-type VHL gene. These isogenic pairs of RCC cell lines, and their baseline expression levels of pVHL and HIF-\(\alpha\) have been widely reported (12, 15). In the second set (ACCH and SN12C), the parental cell line expresses endogenous wild-type VHL\(^{+}\). Isogenic partners that are pVHL-deficient (VHL\(^{low}\)) have been created by stable transduction of VHL-specific shRNA, as described (Supplementary Fig. S1; ref. 14). Compared with VHL\(^{+}\) cells, all pVHL-deficient cell lines (5/5) manifested heightened constitutive JNK/AP1 activity by

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multiple complementary assays: JNK in vitro kinase assays, expression of phosphorylated (Ser73) c-Jun (p-c-Jun), phospho-JNK expression, AP1-driven reporter gene activity, and binding of nuclear extracts to a consensus AP1 response element (i.e., an AP1 DNA binding site) in electrophoretic mobility shift assays (EMSA; Fig. 1C–F). Electrophoretic mobility supershift assays (EMSSA) established that heterodimers of c-Jun and c-Fos, another AP1 family member, represent the components of the AP1 complex that form in the setting of pVHL deficiency (Fig. 1F, a, lanes 5–7).

We next investigated our isogenic cell models for potential interactions between HIF-α and JNK/AP1 activity. Expression
of functionally active pVHL-resistant mutants of HIF-2α in 786-0-VHL− cells did not induce JNK activation (Fig. 2A; refs. 14, 15). Moreover, HIF-1α or HIF-2α suppression with HIF-α–specific siRNA did not reduce p-c-Jun (Ser73) levels in pVHL-deficient cells (Fig. 2B). Conversely, RNAi of c-Jun did not influence HIF-1α or HIF-2α expression (Fig. 2C). Pharmacologic inhibition of JNK with SP600125 (hereafter termed JNKi), did not affect endogenous HIF-1α or HIF-2α expression, in pVHL-deficient UMC6 or UOK121 and 786-0 cells, respectively, but did reduce JNK activity and p-c-Jun levels (Fig. 2D and Supplementary Fig. S2A). These findings implicate pVHL-dependent, HIF-α–independent biochemical signaling events in the JNK/AP1 activation that occurs in the context of pVHL deficiency.

In addition to the effects of the JNKi on p-c-Jun levels described above (Supplementary Fig. S2A), RNA interference (RNAi) of JNK by transduction of a lentivirus expressing JNK-specific shRNA also markedly reduced c-Jun Ser73 phosphorylation (Supplementary Fig. S2B), and the JNKi reduced AP1 reporter activity in a dose-dependent fashion (Supplementary Fig. S2C). These results confirm that JNK is the operative MAPK that regulates c-Jun and AP1 activity in our pVHL-deficient models.

**In vitro and in vivo growth of pVHL-deficient cells is dependent upon constitutive JNK activity**

We next explored the biologic significance of JNK hyperactivity on *in vitro* and *in vivo* growth. The JNKi inhibited the growth of pVHL-deficient but not VHL+ cells *in vitro* in a dose-dependent manner (Fig. 3A). ACHN-VHL− xenografts, which exhibited heightened expression of phosphorylated c-Jun (Fig. 3B, top), grew more rapidly than the VHL+ counterparts in nude mice (Fig. 3B, bottom). Inhibition of JNK expression by transduction of lentivirus expressing JNK-specific shRNA largely abrogated the heightened tumorigenesis observed in pVHL-deficient ACHN cells. In a similar fashion, the JNKi prevented the growth of pVHL-deficient ACHN cells (Fig. 3D).

Next, we confirmed the role of JNK in the tumorigenesis of UOK121 cells, which manifest endogenous VHL inactivation and exhibit intrinsically more delayed and slower tumorigenesis (3–5). Approximately 7 to 8 weeks after tumor cell inoculation to allow for the formation of established tumors, mice harboring subcutaneous UOK121-VHL− xenografts were treated with the JNKi or vehicle control. Pharmacologic JNK inhibition prevented the further growth and actually induced regression of established pVHL-deficient UOK121 xenografts (Fig. 3E).

**Identification of MKK4 and TAK1 as the MAPKK and MAPKKK, respectively, that drive JNK activity in pVHL-deficient RCC cells**

JNK activation is regulated by the MAPKKs, MKK4, and/or MKK7 (21, 22). MKK7 activation status, as determined by phosphorylation of MKK7, was similar between pVHL-deficient and pVHL-expressing cell lines (Fig. 4A). In contrast, phospho-MKK4 expression was increased in pVHL-deficient cell lines (Fig. 4A). Inhibition of endogenous MKK4 activity by ectopic expression of a dominant negative MKK4 construct suppressed AP1 activity (Fig. 4B), a finding that implicates MKK4 as the essential MAPKK in this pathway.

A major branch point in the upstream activation of the JNK signaling axis occurs at the level of the MAPKKs, including MEKK1, ASK1, MLK, and others (10). We focused on TGF-β activating kinase-1 (TAK1), a MAPKKK that not only can function in the JNK pathway but also operates in the NF-κB pathway, which has been previously linked to VHL inactivation (6, 12, 15, 23). Indeed, elevated constitutive TAK1 activation, as measured by TAK1 *in vitro* kinase assays and phosphorylated TAK1 levels, was observed in all (5/5) pVHL-deficient cell lines (Fig. 4C and D). Exposure of pVHL-deficient cells to a pharmacologic TAK1 inhibitor (TAK1i), (5Z)-7-oxozeaenol, reduced MKK4 levels (Fig. 4E), inhibited JNK activity (Fig. 4F), and reduced AP1 reporter activity (Fig. 4H). In addition, TAK1-specific shRNA inhibited JNK activity (Fig. 4G), Taken together, these results identify TAK1 → MKK4 → JNK → c-Jun as a MAPK signaling cascade that is activated in response to VHL inactivation.

**pVHL and CARD9-Dependent TRAF6 lysine 63 polyubiquitination drives TAK1 → JNK activity**

pVHL facilitates an inhibitory phosphorylation of the CARD9 by casein kinase 2 (CK2) in a HIFα-independent

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**Figure 2.** JNK/AP1 activation is HIF-α independent. A, *in vitro* kinase assays showing that expression of a pVHL-resistant HIF-1α or HIF-2α in VHL− cells does not induce JNK activity. B, siRNA-mediated silencing of HIF-1α or HIF-2α in pVHL-deficient cells does not affect p-c-Jun expression. C, siRNA-mediated silencing of c-Jun in pVHL-deficient cells does not affect HIF-α expression. D, pharmacologic inhibition of JNK (4-hour drug exposure) inhibits phosphorylation of c-Jun in a dose-dependent fashion but does not influence expression of HIF-1α or HIF-2α in pVHL-deficient cells.
fashion, and, consequently, in the context of \textit{VHL} loss, there is constitutive activation of CARD9, an upstream trigger for NF-κB activation (6). Upon activation, CARD9 typically forms a multiprotein complex with other proteins including BCL10 and Malt1 (24), which in turn recruit TRAFs (TNF receptor–associated factors), that subsequently oligomerize and transautoubiquitinate. The latter process results in lysine 63 (K63)-linked polyubiquitination, which, unlike K48 polyubiquitin linkages that classically result in proteasome-dependent degradation, is a proteasome-independent, activating event.

Figure 3. JNK blockade inhibits the growth of pVHL-deficient RCCs. A, in vitro growth of pVHL-deficient but not pVHL-replete cells is inhibited by pharmacologic JNK blockade. Cells were exposed to the JNKi for 120 hours before assessing overall cell viability by MTT assay. Results were normalized to that of vehicle-treated controls and are means of 8 experiments ± SD. B, pVHL deficiency leads to rapid tumorigenesis that is associated with AP1 activation. After SN12C cells were inoculated subcutaneously into the flanks of nude mice (\(n = 6\)/group), tumor volume was measured weekly. Results are averages ± SD. \(^\ast\), \(P = 0.012\). Top, Western blotting on protein extracts obtained from frozen xenografts harvested at the time of animal sacrifice. C, tumorigenesis of ACHN-VHLlow cells transduced with JNK-specific or NS shRNA (\(n = 6\)/group). \(^\ast\), \(P = 0.0066\); \(^\ast\ast\), \(P = 1.8 \times 10^{-5}\). Inset, JNK Western blotting on protein extracts obtained 48 hours after lentiviral transduction. Untransduced cells serve as a control for JNK expression. D, pharmacologic inhibition of JNK prevents tumor progression of ACHN-VHLlow xenografts. Once subcutaneous tumors reached a volume of approximately 50 mm³, animals (\(n = 6\)/group) were treated with 25 mg/kg of the JNKi by i.p. injection every other day, and tumor volume was monitored. \(^\ast\), \(P = 0.0011\). E, tumorigenesis of UOK121-VHL+ cells is markedly delayed by JNKi administration. When subcutaneous xenografts (\(n = 6\)/group) became palpable, mice were treated with JNKi (12.5 mg/kg i.p. twice per week). \(P < 0.001\). Results are means of tumor volumes ± SD.
activated TRAFs recruit and activate TAK1 (in complex with the adaptor proteins TAB1/2). Thus, we postulated that VHL inactivation is linked to TAK1 through disinhibition of CARD9 followed by a CARD9- and TRAF-dependent activation and recruitment of TAK1.

We initially evaluated the ubiquitination state of TRAF6, which is known to function upstream of TAK1 (25). pVHL−deficient cells showed appreciably more constitutive TRAF6 ubiquitination (Fig. 5A); the TRAF6 polyubiquitination was mediated through K63 linkages (Fig. 5B). Transient transfection of a TRAF6 dominant negative resulted in a dose-dependent reduction in AP1-driven reporter gene expression in pVHL−deficient cells, a finding that mechanistically links TRAF6 to the JNK/AP1 pathway (Fig. 5C).

Next, we postulated that CARD9−TRAF6 interactions form more robustly in pVHL−deficient cells. Indeed, appreciably higher amounts of CARD9 coimmunoprecipitated with TRAF6 in all pVHL−deficient cells compared with their VHL+ counterparts (Fig. 5D, top) despite the fact that VHL+ cells constitutively express higher levels of CARD9 (Fig. 5D, bottom). TRAFs deliver their downstream signals in the context of protein complexes that contain not only CARD9 but also other modulatory proteins, such as BCL10. In fact, in pVHL−deficient cells, more BCL10 coimmunoprecipitated with TRAF6 compared with VHL+ cells (Fig. 5D, top), yet BCL10 expression did not vary in a pVHL-dependent fashion (Fig. 5D, bottom).

These biochemical findings suggested to us that mitigation of the inhibitory phosphorylation of CARD9 by CK2 in the context of VHL inactivation, allows for the formation of a complex that includes CARD9, BCL10, and TRAF6, which serves as a proximal signal for TAK1 and ultimately JNK/AP1 activation. To directly test this notion, we transfected cells that endogenously express wild-type pVHL (ACHN and SN12C) with a wild-type myc-tagged CARD9 construct or a phospho-decient mutant of the CARD9 construct in which all CK2 phosphorylation sites were mutated to alanine, and is thus resistant to the CK2-dependent inhibitory phosphorylation. As predicted, the phospho-decient CARD9 mutant more readily coimmunoprecipitated with TRAF6 (Fig. 5E), indicating that the phosphorylation of CARD9 by CK2 functions to inhibit the formation of a CARD9−TRAF6 complex.

To determine whether the CARD9−TRAF6 interaction drives the activation of the JNK signaling axis in a pVHL−deficient context, we conducted RNA interference of CARD9 and examined the downstream effects on TRAF6 K63 polyubiquitination and TAK1 activation. CARD9 silencing led to a sharp reduction in TRAF6 K63 polyubiquitination in pVHL−deficient cells (Fig. 5F and G) and inhibited AP1 reporter activity as well as TAK1 activation. These findings suggest that VHL−deficient cells lose inhibitory phosphorylation at CK2-dependent sites on CARD9, allowing for the formation of a CARD9−TRAF6 complex that serves as a proximal signal for TAK1 and JNK/AP1 activation.
Figure 5. VHL inactivation promotes CARD9/TRAF6 complex formation and TRAF6 K63 ubiquitination. A, increased TRAF6 ubiquitination in pVHL-deficient cells. B, increased TRAF6 ubiquitination occurs through K63 in pVHL-deficient cells. C, TRAF6-DN inhibits AP1 reporter activity. Results are means of triplicates. D, increased TRAF6 immunoprecipitation with CARD9 and BCL10 in pVHL-deficient cells. Bottom, Western blotting. Top, coimmunoprecipitation studies. E, mutagenesis of the CK2 phosphorylation sites within CARD9 promotes the interaction between CARD9 and TRAF6. Bottom, Western blotting shows equivalent expression of the wild-type and mutant CARD9 proteins. Top, coimmunoprecipitation studies. F, CARD9 siRNA reduces TRAF6 K63 ubiquitination. 786-0-VHL cells were transiently transfected with one of 2 CARD9 siRNAs. Top, Western blotting. Bottom, CARD9 siRNA reduces TRAF6 K63 polyubiquitination. G, Same as F but assay also conducted on additional cell lines with CARD9 siRNA (C9-1). H, CARD9 siRNA inhibits AP1 reporter activity. Results are means of triplicates. I, schematic of biochemical pathway whereby pVHL deficiency leads to JNK/AP1 activation.
kinase activity (Fig. 5H and Supplementary Fig. S3). In summary, the results of our biochemical studies along with previously published work indicate that pVHL deficiency results in the disinhibition of CARD9 (6), formation of a protein complex amongst CARD9, BCL10, and TRAF6, and activation of TRAF6 through K63 polyubiquitination, which triggers the sequential activation of TAK1, MKK4, and JNK (Fig. 5I).

JNK/AP1 Induces an EMT phenotype mediated by Twist

We previously reported differences in cellular morphology between VHL+ and VHLlow ACHN and SN12C cells (14). Whereas VHL+ cells grew in cellular clusters with individual cells taking on a polygonal shape, VHLlow cells often grew as single cells that manifested an elongated, fibroblastic morphology consistent with an epithelial–mesenchymal transition (EMT). We now report differences in EMT characteristics and the role of JNK/AP1 and Twist in this process.

Figure 6. An EMT occurring in the context of VHL inactivation is dependent upon JNK/AP1 activation of Twist expression. A, morphology of VHL+ and VHLlow cells. Scale bar, 100 μmol/L. B, Western blotting shows a mesenchymal pattern of cadherin expression and increased Twist and Slug expression in pVHL-deficient cells. C, heightened invasiveness of pVHL-deficient cells in a Matrigel chamber invasion assay. Results are means of cells counts in 3 × 100 fields ± SD. Scale bar = 100 μmol/L. *, P = 0.00024; **, P = 0.00058. Comparisons are VHL+ versus VHLlow cells. Importantly, cell proliferation of pVHL-deficient and VHL+ cells does not differ over the time course of the Matrigel invasion assay. D, Western blotting showing effects of c-Jun siRNA on expression of cadherins and indicated transcription factors in pVHL-deficient cells. E, same as D but JNK shRNA in lieu of c-Jun siRNA. F, dose-dependent effects of JNKi on expression of indicated proteins (Western blots). G, c-Jun siRNA reduces invasiveness of pVHL-deficient RCCs in Matrigel chamber assay. *, P = 0.00041; **, P = 0.00012. H, JNKi reduces invasiveness of pVHL-deficient cells.

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Figure 7. Twist expression induces JNK-dependent EMT. A, Twist siRNA inhibits invasiveness of pVHL-deficient ACHN and SN12C cells. a, Western blotting shows effective silencing of Twist. b, effect of Twist silencing on invasiveness. *P = 0.0068; **P = 0.0031; ***P = 0.0011; ****P = 0.0002. c, light micrographs of cells that have invaded into Matrigel. Scale bar, 100 μm. B, same as A but in 786-0-VHL cells. *P = 0.00070; **P = 0.00039. C, ectopic Twist (eTwist) expression restores invasiveness of JNKi-treated VHLlow ACHN and SN12C cells. Cells were transduced with lentiviral particles expressing eTwist or red fluorescent protein, and, 72 hours later, were analyzed in a Matrigel invasion assay with or without the JNKi (10 μmol/L for 24 hours). n.s., not statistically significant for comparisons of red fluorescent protein transduced + vehicle treated to Twist transduced + JNKi treated; P = 0.37 and P = 0.99 for ACHN and SN12C, respectively. D, Western blotting for indicated proteins show that eTwist expression prevents the induction of E-cadherin by JNKi and that eTwist-expressing cells exposed to the JNKi (10 μmol/L) have similar total Twist expression as red fluorescent protein-expressing, vehicle-treated cells. E, schematic figure of the Twist gene and promoter (modified from NCBI database). Approximately 2.5 kb upstream of the transcription start site was analyzed for putative AP1 binding sites with the aid of the M-Match search engine, and 5 PCR primers sets spanning predicted AP1 sites were generated for ChIP analysis (see Supplementary Table S2). F, ChIP analysis of Twist promoter. PCR products (Supplementary Table S2) from sets 1, 2, and 3 are shown.
(EMT; Fig. 6A). As reported (14), VHL<sup>low</sup> cells also manifested a "cadherin switch," whereby the characteristic pattern of increased E-cadherin and reduced N-cadherin expression typified by epithelial cells and observed in VHL<sup>+</sup> cells transitioned to a mesenchymal pattern of heightened N-cadherin and suppressed E-cadherin expression (Fig. 6B and Supplementary Fig. S4A). Moreover, compared with VHL<sup>+</sup> cells, pVHL-deficient cells showed markedly heightened invasiveness in a Matrigel chamber, a finding that further supports the notion of EMT induced by pVHL deficiency (Fig. 6C and Supplementary Fig. S4B) that has been described (26–28). Although these prior studies have implicated HIF-α as a pivotal factor in the EMT observed in pVHL-deficient RCCs, we investigated the potential for JNK/AP1 to function in a parallel pathway to mediate EMT in this context.

RNAi with c-Jun–specific siRNA reversed the mesenchymal cadherin expression pattern in VHL<sup>low</sup> cells (Fig. 6D). Similar effects on cadherin expression were observed when JNK-specific shRNA was introduced (Fig. 6E). In pVHL-deficient cells, the JNKi induced a dose- and time-dependent reversion of the "cadherin switch" back to that of VHL<sup>+</sup> cells (Fig. 6F and Supplementary Fig. S5). Similarly, the heightened invasiveness of pVHL-deficient cells was reversed by the JNKi as well as c-Jun–specific siRNA (Fig. 6G and Supplementary Fig. S6); the JNKi did not influence the invasiveness of VHL<sup>+</sup> cells (not shown). JNK inhibition did not result in a reversion of the morphology of pVHL-deficient cells from a mesenchymal to epithelial phenotype, a finding that implicates JNK-independent effects on cellular morphology.

We screened for differential expression of transcription factors, including Twist, Slug, Snail, Zeb1, and Zeb2, which are known to regulate EMT and E-cadherin expression (29–31). Twist and Slug expression was augmented in pVHL-deficient cells compared with their VHL<sup>+</sup> counterparts (Fig. 6B and Supplementary Fig. S4), whereas the expression of Zeb1, Zeb2, and Snail was unaffected by VHL status (Fig. 6B and Supplementary Fig. S4). RNAi of c-Jun sharply decreased the augmentation of Twist expression in VHL<sup>low</sup> cells but had no effect on Slug or Snail expression (Fig. 6D). In the same manner, the JNKi reduced Twist, but not Slug or Snail expression, in both a dose- and time-dependent fashion (Fig. 6F and Supplementary Fig. S5), as did lentiviral-mediated introduction of JNK-specific shRNA (Fig. 6E).

Suppression of Twist expression by Twist siRNA reduced the invasiveness of pVHL-deficient cells (Fig. 7A and B). Ectopic Twist expression driven by the AP1-independent CMV promoter in pVHL-deficient cells was sufficient to overcome the inhibitory effects of the JNKi on invasion and E-cadherin expression (Fig. 7C and D). Taken together, these findings indicate that the mesenchymal phenotype of pVHL-deficient cells is dependent upon JNK-induced Twist expression, although JNK-independent pathways mediated by HIF-α have also been described and may be operative (26–28).

c-Jun/c-Fos Heterodimers transactivate Twist through an upstream AP1 cis-acting element

We next investigated the transcriptional regulation of Twist expression by AP1. A virtual analysis of the 2.5 kb region upstream of the Twist transcription start site identified numerous potential AP1 binding sites with varying degrees of homology to the consensus AP1 sequence (Fig. 7E and Supplementary Table S2). Using chromatin immunoprecipitation (ChIP) experiments, we identified a 350 bp region (−2,589→−2,238 relative to the transcription start site), hereafter termed the set 1 segment, that contains 4 predicted AP1 binding sites to which c-Jun was recruited (Fig. 7F). On the basis of the EMSSAs shown in Fig. 1F, we predicted that c-Jun forms heterodimers with c-Fos on the AP1 DNA binding sites of the Twist promoter. Indeed, ChIP experiments confirmed that c-Fos was recruited to the set 1 region of the Twist promoter in a similar manner as c-Jun (Fig. 7F).

To determine whether the set 1 region manifests functional activity, we cloned the set 1 region into a firefly luciferase reporter construct. Increased reporter gene activity driven by the set 1 segment of the Twist promoter was observed in VHL<sup>low</sup> cells compared with VHL<sup>+</sup> cells (Fig. 7G). In contrast, a similarly sized segment of the Twist promoter, which did not show AP1 binding in ChIP assays (set 3, Fig. 7E), had shown similar reporter gene activity in VHL<sup>low</sup> and VHL<sup>+</sup> cells (Fig. 7G). Differential reporter gene activity attributable to the set 1 segment was inhibited by exposure of VHL<sup>low</sup> cells to the JNKi (Fig. 7H). Taken together, our results indicate that an AP1 complex, composed of c-Jun/c-Fos heterodimers, binds to AP1 response elements in the regulatory region upstream of the Twist promoter, thereby driving Twist protein expression.

Discussion

We have provided several lines of evidence that pVHL deficiency causally results in JNK activation, whereby the inhibitory phosphorylation of CARD9 by CK2 is hindered by the inactivation of VHL (6), CARD9 forms a complex with TRAF6 and BCL10, resulting in TRAF 6 activation followed in sequence by TAK1, MKK4, and JNK activation. Importantly, tumorigenesis of pVHL-deficient RCCs is dependent upon JNK, which may represent a suitable biochemical target for drug discovery efforts. The finding of frequent expression of nuclear p-c-Jun, a substrate of JNK, in clear cell RCCs further validates JNK as a target for clinical translation.

Given that the JNKi rescued the JNK-dependent reporter gene activity, we predicted that AP1 might regulate Twist transcription in a JNK-dependent manner. The ChIP experiments identified the AP1 binding sites at the Twist promoter that were conserved in a human RCC band that bound JNK. Furthermore, we have previously shown similar reporter gene activity in VHL<sup>low</sup> and VHL<sup>+</sup> cells (Fig. 7E). Taken together, our findings that implicates JNK-independent effects on cellular morphology.
We and others have observed that VHL inactivation can induce a mesenchymal phenotype (14, 26–28). In the process of elucidating the molecular underpinnings that drive EMT in pVHL-deficient RCCs, we found that JNK/AP1 induces Twist transcription. Specifically, AP1 complexes composed of c-Jun and c-Fos heterodimers transcriptionally upregulate Twist expression by directly binding to and transactivating AP1 cis-acting elements upstream of the transcription start site of the Twist gene. To our knowledge, JNK/AP1 has not been previously reported to regulate expression of Twist. As a consequence of increased Twist expression, pVHL-deficient cells manifest reduced E-cadherin and increased N-cadherin expression as well as other characteristics of EMT, including increased invasiveness and a fibroblastic morphology.

Previous reports have found that EMT and reduced E-cadherin expression in pVHL-deficient cells occurs in an HIF-α-dependent manner (26–28). For example, one group found that HIF-1α induces Twist protein expression by binding to hypoxia response elements in the Twist promoter (32). Some reports specifically indicated HIF-1α as the principal mediator of EMT (28), others point to HIF-2α (27), whereas HIF-1α and HIF-2α were found to function in this regard by yet others (26). No clear explanation for these discrepancies can be readily discerned from the methodologies in these studies, although some of the differential results may be partially accounted for by the use of different cell lines. Nonetheless, our results that EMT regulatory transcription factors and EMT itself are regulated in a JNK/AP1-dependent fashion are not mutually exclusive with HIF-α-dependent regulation of these same processes. In point of fact, it seems that HIF-α as well as JNK can both transcriptionally upregulate the expression of transcription factors that mediate EMT, which supports the notion that JNK and HIF-α function in parallel to coordinate drive RCC growth and tumorigenesis.

On the basis of the finding of somatic VHL allele inactivation observed in the premalignant renal cysts of patients with von Hippel–Lindau syndrome, the biallelic inactivation of VHL is thought to represent an early mutational event in renal carcinogenesis (33, 34). Accordingly, we postulate that not only constitutive HIF-α expression but also JNK activation represents an early molecular event that occurs in response to VHL inactivation. Additional acquired genetic lesions, which have only been partially identified and elucidated but include mutations in genes that regulate chromatin remodeling and structure, accumulate and are required to initiate RCC (35, 36).

In summary, we have presented several lines of evidence that JNK/AP1 is constitutively activated in the pVHL-deficient state. pVHL deficiency leads to dependence on the activity of nonmutated JNK for proliferation and tumorigenesis in a state of nononcogene addiction. This dependence of clear cell RCCs on the kinase activity of JNK can potentially be exploited for clinical translation. Thus, JNK/AP1 hyperactivation may represent a nononcogene addiction target, and further optimization of commercially available JNK inhibitors may constitute a viable endeavor for clinical application to the treatment of clear cell RCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. An, M. Veena, E. Srivatsan, M.B. Rettig
Development of methodology: J. An, H. Liu, J. Huang, M.B. Rettig
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